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Tonic ubiquitylation controls T-cell receptor:CD3 complex expression during T-cell development

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Expression of the T-cell receptor (TCR):CD3 complex is tightly regulated during T-cell development. The mechanism and physiological role of this regulation are unclear. Here, we show that the TCR:CD3 complex is constitutively ubiquitylated in immature double positive (DP) thymocytes, but not mature single positive (SP) thymocytes or splenic T cells. This steady state, tonic CD3 monoubiquitylation is mediated by the CD3ε proline-rich sequence, Lck, c-Cbl, and SLAP, which collectively trigger the dynamin-dependent downmodulation, lysosomal sequestration and degradation of surface TCR:CD3 complexes. Blocking this tonic ubiquitylation by mutating all the lysines in the CD3 cytoplasmic tails significantly upregulates TCR levels on DP thymocytes. Mimicking monoubiquitylation by expression of a CD3ζ-monoubiquitin (monoUb) fusion molecule significantly reduces TCR levels on immature thymocytes. Moreover, modulating CD3 ubiquitylation alters immunological synapse (IS) formation and Erk phosphorylation, thereby shifting the signalling threshold for positive and negative selection, and regulatory T-cell development. Thus, tonic TCR:CD3 ubiquitylation results in precise regulation of TCR expression on immature T cells, which is required to maintain the fidelity of T-cell development.

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Introduction

The T-cell receptor (TCR):CD3 complex, one of the most complicated receptor complexes on the cell membrane, is composed of a TCRαβ heterodimer, which recognizes cognate antigen, and the associated CD3 molecules (CD3δ, γ, ε, and ζ), which are required for TCR expression, signal transduction, and receptor transport (Clevers *et al*, 1988; Klausner *et al*, 1990). A hallmark of T-cell development in the thymus is the tightly controlled cell surface expression of the TCR:CD3 complex, which is low on double positive (DP; $CD4^+CD8^+$) thymocytes and high on single positive (SP; $CD4^+$ or $CD8^+$) thymocytes and peripheral T cells (Bluestone *et al*, 1987). Subtle changes in TCR signal strength determine the fate of immature thymocytes and therefore the fine control of TCR:CD3 expression level on immature thymocytes is likely to be essential in controlling T-cell selection (Daniels *et al*, 2006). However, the precise mechanism that controls TCR expression in the thymus is unclear. Initially, a posttranscriptional mechanism or instability of the nascent TCRα protein was proposed to regulate TCR:CD3 expression during T-cell development (Bluestone *et al*, 1987; Klausner *et al*, 1990; Kearse *et al*, 1994, 1995). However, recent studies have suggested that ubiquitylation may contribute to this process. Ubiquitin (Ub) can be attached to proteins via free lysine (K) residues by specific E3 Ub ligases (Glickman and Ciechanover, 2002). Monoubiquitylation (the addition of a single Ub residue) has been shown to modulate protein endocytosis and intracellular transport (Haglund *et al*, 2003a; Marmor and Yarden, 2004). MonoUb can be further modified to generate polyUb chains via an isopeptide linkage between the C-terminal glycine carboxyl group of the Ub being added and the ε-amino group of a lysine on the protein-attached Ub (Hochstrasser, 2006), which predominantly occurs at K48 and K63 (Peng *et al*, 2003; Xu and Peng, 2008). Whereas K48-linked polyubiquitylation typically leads to protein degradation by 26S proteasomes (Glickman and Ciechanover, 2002; Goldberg, 2003), K63-linked polyubiquitylation is thought to modulate a variety of events including signal transduction and receptor endocytosis (Wang *et al*, 2001). Two molecules that are highly expressed in the thymus, c-Cbl (*Cbl*; Casitas B-lineage lymphoma), a RING-type E3 Ub ligase, and SLAP (*Sla*; Src-like adaptor protein), a c-Cbl adaptor protein, are thought to modulate TCR expression on

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DP thymocytes (Sosinowski *et al*, 2001; Molero *et al*, 2004; Myers *et al*, 2006). However, c-Cbl and SLAP interact with and/or ubiquitylate multiple T-cell signalling molecules, such as Lck and Zap70 (Tang *et al*, 1999; Thien and Langdon, 2001; Rao *et al*, 2002), and thus it is unclear which interaction is responsible for the regulation of TCR expression on immature T cells. Recently, it was suggested that LAPTM5, a lysosomal protein, negatively regulates surface TCR expression by degrading CD3ζ via its Ub-interacting motif, suggesting a possible role for ubiquitylation in modulating TCR:CD3 complex expression (Ouchida *et al*, 2008). However, TCR expression on DP thymocytes was only slightly increased in *Laptm5*$^{-/-}$ mice.

In this study, we addressed the following questions using biochemical analysis, confocal microscopy, and *in vivo* genetic approaches: (1) Is the TCR:CD3 complex differentially ubiquitylated in thymic and splenic T cells, and if so what type of ubiquitylation occurs (e.g. monoUb, polyUb)? (2) What is the mechanism by which TCR:CD3 surface expression is regulated and what molecules participate in CD3 ubiquitylation? (3) What is the physiological role of TCR:CD3 ubiquitylation?

Results

Tonic ubiquitylation of CD3 in DP thymocytes

To investigate the mechanism that regulates TCR surface expression on immature T cells (Supplementary Figure S1A), we first confirmed that CD3 protein expression in DP thymocytes is much lower than SP thymocytes (Supplementary Figure S1B), which is clearly due to a post-transcriptional degradative event, as mRNA levels of all four CD3 molecules were similar between DP and SP thymocytes (Supplementary Figure S1C) (Maguire *et al*, 1990).

As some receptors have been shown to be regulated by a Ub-dependent endocytosis mechanism (Shih *et al*, 2000), and CD3ζ and δ are ubiquitylated after T-cell activation (Cenciarelli *et al*, 1992), we speculated that increased ubiquitylation of CD3 may occur in immature thymic T cells. To test this possibility, we assessed whether CD3ζ was ubiquitylated in DP thymocytes. CD3ζ from resting SP thymocytes, DP thymocytes, and splenic T cells was immunoprecipitated, separated on an SDS–PAGE gel and western blots probed with a Ub monoclonal antibody (mAb). Lysate from cross-linked anti-CD3ε-activated splenic T cells was included as a positive control. As expected, we observed a ladder of ubiquitylated CD3ζ in activated, but not resting, splenic T cells. The size of these bands (24, 32, 40 kD, etc.) was consistent with CD3ζ modification by progressive addition of the 8-kD Ub protein (Cenciarelli *et al*, 1992). Remarkably, constitutively ubiquitylated CD3ζ was observed in DP, but not SP thymocytes in the absence of TCR:CD3 crosslinking (Figure 1A). Similarly, we detected ubiquitylation of CD3ε in DP, but not SP thymocytes following immunoprecipitation with CD3ε mAbs and western blot analysis with a Ub mAb (Figure 1B). A comparable polyUb ladder was also seen by direct western blot analysis of DP thymocyte lysates with a CD3ζ mAb (Figure 1C). These data show that at any one time only a small proportion of CD3ζ is ubiquitylated suggesting a dynamic process.

Given that CD3ζ in splenic T cells is ubiquitylated following TCR ligation, it is possible that the observed ubiquitylation in DP thymocytes is derived from the fraction of thymocytes that are undergoing positive selection. Interestingly, a similar level of CD3ζ ubiquitylation was observed in CD69^{lo} prepositive selection DP thymocytes and DP thymocytes from mice lacking MHC class I and class II [*B2m*$^{-/-}$*Abb*$^{-/-}$] (Figure 1D). These data suggest that CD3ζ ubiquitylation in DP thymocytes is constitutive and MHC-ligation independent, showing that this is not restricted to cells undergoing positive selection. Hereafter, we refer to this process as 'tonic' ubiquitylation as it appears analogous to the low level, constitutive tyrosine phosphorylation of the TCR:CD3 observed in DP thymocytes (Nakayama *et al*, 1989). *Cbl*$^{-/-}$ DP thymocytes express high levels of TCR (Figure 1E) (Murphy *et al*, 1998; Naramura *et al*, 1998), suggesting c-Cbl is involved in TCR downregulation. Indeed, the *Cbl*$^{-/-}$ thymocytes exhibit little CD3ζ ubiquitylation showing that c-Cbl expression is required for ubiquitylation of CD3ζ (Figure 1F).

Given that ubiquitylation has been shown to alter protein trafficking and intracellular location (Haglund *et al*, 2003a; Marmor and Yarden, 2004; Mukhopadhyay and Riezman, 2007), we questioned whether the TCR:CD3 complex was differentially distributed in SP versus DP thymocytes. In wild-type mice, CD3ζ was uniformly distributed on the cell surface of SP thymocytes (Figure 1G and H). Surprisingly, although some CD3ζ in DP thymocytes was expressed on the cell surface, most appeared to be confined to a single, defined intracellular compartment. A similar differential staining pattern was observed when thymocytes were stained with antibodies to either TCRβ or CD3ε (data not shown). Interestingly, this TCR:CD3 sequestration was not observed in *Cbl*$^{-/-}$ DP thymocytes, which instead looked essentially identical to the even surface staining pattern observed in wild-type SP thymocytes and their *Cbl*$^{-/-}$ SP counterparts (Figure 1G and H). These data show that CD3 ubiquitylation leads to the redistribution and intracellular sequestration of the TCR:CD3 complex in DP thymocytes. We also observed a similar phenotype in *Sla*$^{-/-}$ thymocytes (Supplementary Figure S2). Taken together, these data suggest that c-Cbl/SLAP-dependent, MHC-independent tonic ubiquitylation of the TCR:CD3 complex occurs on DP thymocytes, but not SP thymocytes or peripheral T cells and that this modulates TCR surface expression by orchestrating its cellular sequestration.

Lck and the CD3ε proline-rich sequence are required for CD3 tonic ubiquitylation

To determine whether Src kinases are involved in the regulation of TCR expression in DP thymocytes, we treated neonatal thymi with Src kinase inhibitors (PP1 and PP2). Inhibition of Src kinase activity upregulated surface TCR expression on DP thymocytes, suggesting that either Lck or Fyn might be involved in the CD3 tonic ubiquitylation pathway (Figure 2A). We next analysed TCR expression on DP thymocytes from WT, *Fyn*$^{-/-}$, and *Lck*$^{-/-}$ mice by flow cytometry. Although Fyn deficiency had no effect on TCR expression on DP thymocytes, there was a substantial increase in TCR expression on *Lck*$^{-/-}$ DP thymocytes (Figure 2B). Likewise the absence of Lck, but not Fyn altered the distribution pattern of CD3ζ in DP thymocytes, which was indistinguishable to that observed in wild-type SP thymocytes (Figure 2C and D). To test whether Lck or Fyn mediates tonic ubiquitylation of CD3, we assessed the ubiquitylation status of CD3ζ in DP thymocytes of WT, *Fyn*$^{-/-}$, and *Lck*$^{-/-}$ mice. Although

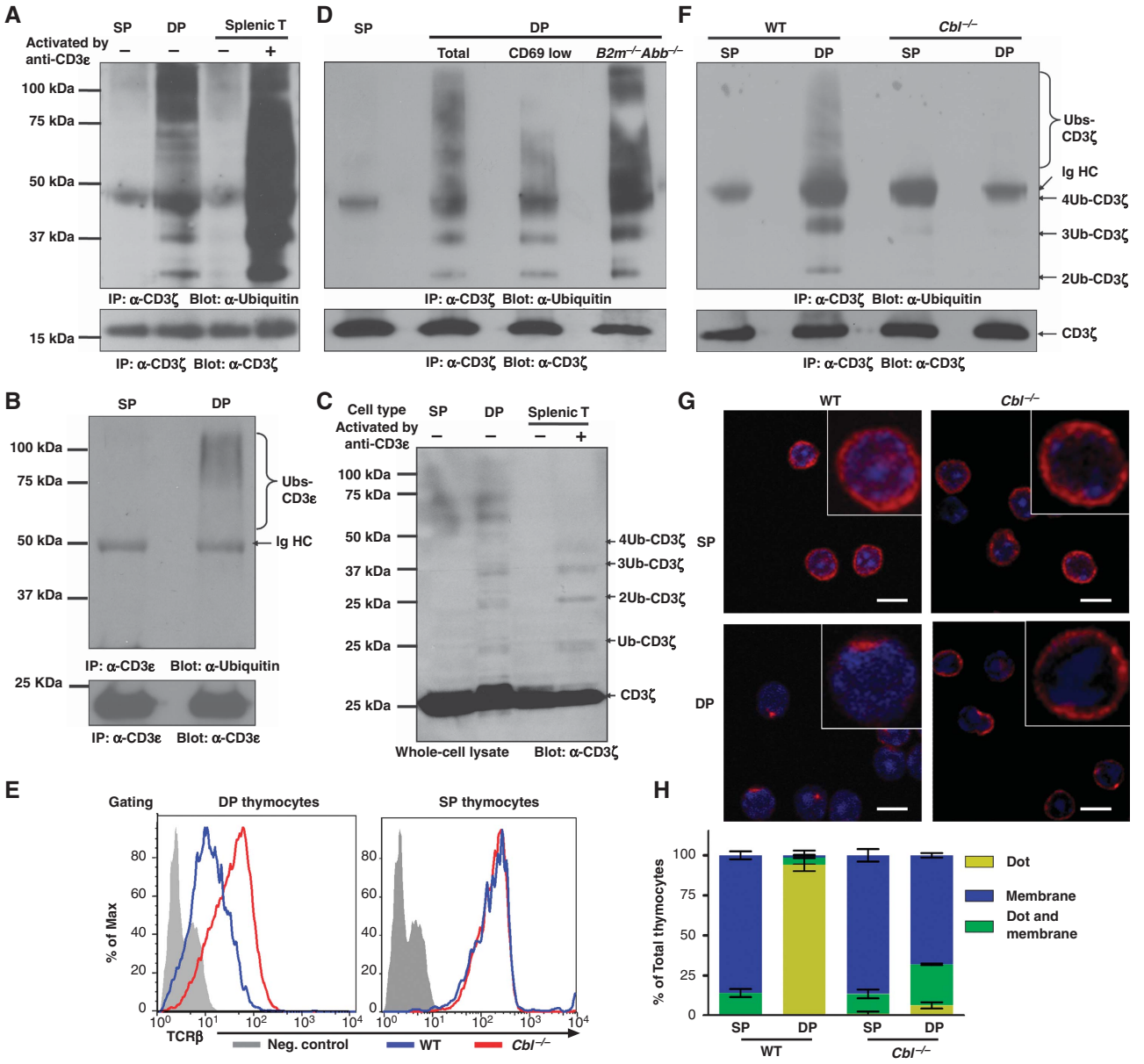

Figure 1 c-Cbl-mediated tonic ubiquitylation of CD3 on immature T cells. (**A**–**D**, **F**) DP and SP thymocytes were purified by FACS, and splenic T cells purified by MACS. Splenic T cells were either resting or activated by crosslinking CD3ε for 2 min. (**A**) SP thymocytes (3×10^7), DP thymocytes (24×10^7), and resting or activated splenic T cells (3×10^7) were lysed in 1 ml lysis buffer. The lysates were immunoprecipitated with CD3ε antisera (551ζ), separated by SDS–PAGE and western blots was probed with Ub mAb (P4D1). Blots were stripped and reprobed with CD3ζ mAb (H146) to show that an equal amount of CD3ζ was analysed in each sample. (**B**) After immunoprecipitation with CD3ζ mAb as described in (**A**), the lysates were further immunoprecipitated with CD3ε mAb (2C11), separated by SDS–PAGE and western blots was probed with Ub mAb. Blots were stripped and reprobed with CD3ε mAb (HTM3.1). (**C**) Lysates (20 μl) were separated by SDS–PAGE and immunoblotted with CD3ζ mAb. (**D**, **F**) Analysis was performed as in (**A**), except that wild-type CD69^{low} and *B2m*$^{-/-}$*Abb*$^{-/-}$ [MHC class I and class II deficient] DP thymocytes (**D**), or *c-Cbl*$^{-/-}$ SP and DP thymocytes (**F**) were purified by FACS and included in the analysis. (**E**) DP and SP thymocytes were purified from either C57BL/6J mice or *c-Cbl*$^{-/-}$ mice, and TCRβ surface expression was measured by flow cytometry. (**G**, **H**) Thymocytes were stained with mAbs against CD4 and CD8, and DP and SP thymocytes purified by FACS. Sorted thymocytes were fixed, permeabilized, and stained with Alexa-647-conjugated CD3ζ mAb. The localization of CD3ζ (red) and DAPI-stained nucleus (blue) are shown in representative confocal images (**G**). The distribution of CD3ζ in thymocyte populations is indicated ($n > 50$) (**H**).

tonic ubiquitylation of CD3ζ was observed in WT and *Fyn*$^{-/-}$ DP thymocytes, it was absent in *Lck*$^{-/-}$ DP thymocytes (Figure 2E). These data show that Lck, rather than Fyn, is required for CD3 tonic ubiquitylation and thus regulation of TCR surface expression on immature T cells.

Earlier studies have shown that the proline-rich sequence (PRS) in CD3ε modulates TCR expression and signal transduction, suggesting that the CD3ε-PRS may be involved in tonic ubiquitylation of CD3 in DP thymocytes (Szymczak *et al*, 2005; Mingueneau *et al*, 2008; Tailor *et al*, 2008). To assess this possibility, we examined CD3 expression, distribution, and ubiquitylation in DP thymocytes from mice possessing a knock-in mutation of the CD3ε PRS (*Cd3e*$^{\Delta PRS/\Delta PRS}$) (Mingueneau *et al*, 2008). As described earlier (Mingueneau *et al*, 2008), TCR expression was increased on *Cd3e*$^{\Delta PRS/\Delta PRS}$ DP thymocytes compared with their wild-

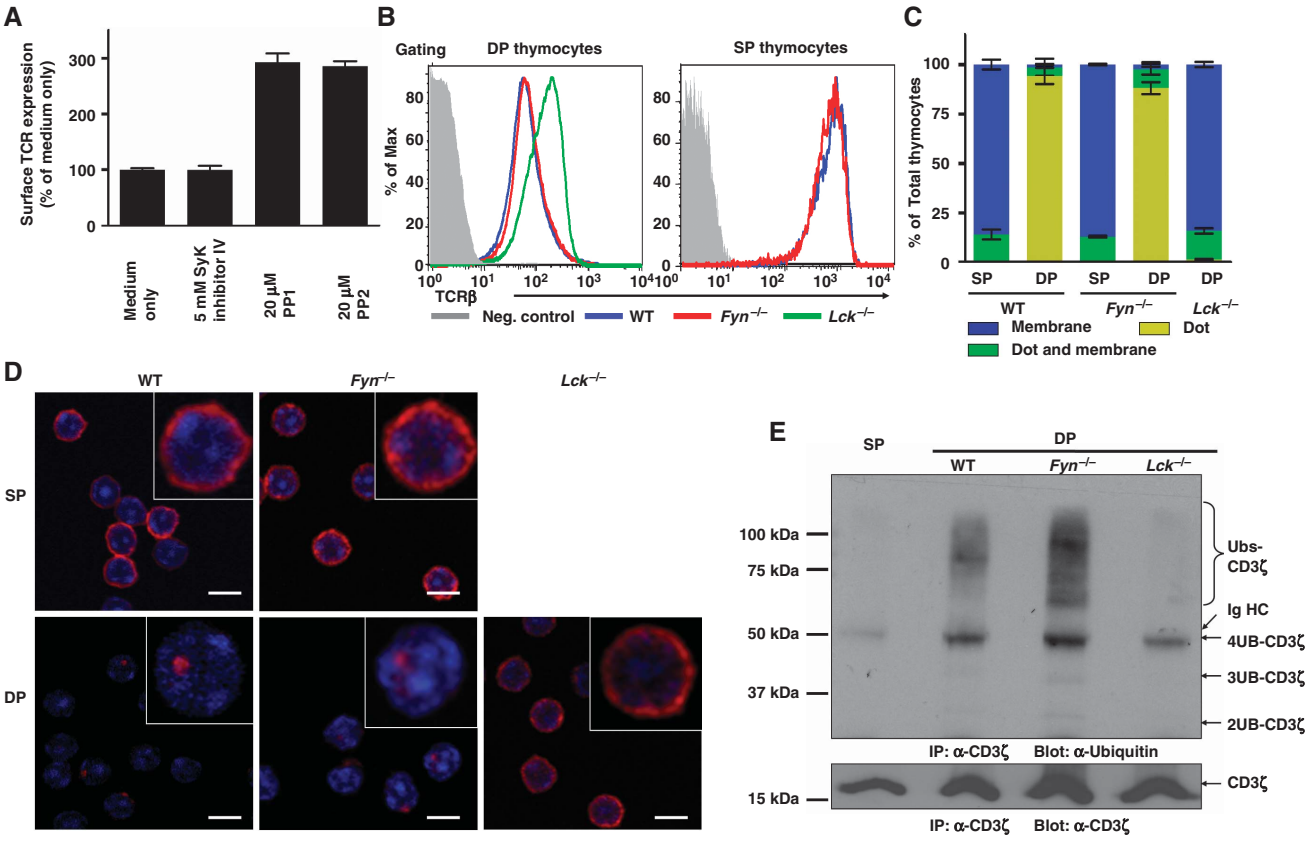

Figure 2 Lck is required for tonic ubiquitylation of CD3 in DP thymocytes. (**A**) Thymic lobes from newborn mice (P1) were treated with the inhibitors indicated for 20 h, and surface TCRβ expression determined by flow cytometry. Data represent the mean ± s.e.m. of 5–10 mice from 2–3 experiments per group. (**B**) Thymi were isolated from WT, *Fyn*$^{-/-}$, or *Lck*$^{-/-}$ mice. Surface expression of the TCR:CD3 complex was measured by flow cytometry on thymocytes co-stained with mAbs against CD4, CD8, and TCRβ. (**C**, **D**) Thymocytes were stained with mAbs against CD4 and CD8, and DP and SP thymocytes purified by FACS. Sorted thymocytes were fixed, permeabilized, and stained with Alexa-647-conjugated CD3ζ mAb. The distribution of CD3ζ in thymocyte populations is indicated ($n > 50$) (**C**). The localization of CD3ζ (red) and DAPI stained nucleus (blue) are shown in representative confocal images (**D**). (**E**) Tonic ubiquitylation of CD3 was performed with WT, *Fyn*$^{-/-}$, or *Lck*$^{-/-}$ mice as described in Figure 1A.

type controls (Figure 3A). As seen in DP thymocytes lacking c-Cbl, Slap, and Lck, DP thymocytes from *Cd3e*$^{\Delta PRS/\Delta PRS}$ mice exhibited an even TCR:CD3 surface distribution, as seen in wild-type SP thymocytes (Figure 3B and C). Furthermore, there was a complete loss of CD3ζ tonic ubiquitylation in *Cd3e*$^{\Delta PRS/\Delta PRS}$ DP thymocytes (Figure 3D). Taken together, these data show that the CD3ε-PRS is required for CD3 tonic ubiquitylation and suggests that CD3ε-PRS association with Nck may mediate TCR:CD3 complex translocation to intracellular compartments.

TCR:CD3 monoubiquitylation mediates its dynamin-dependent internalization and targeting to lysosomes for degradation

The intracellular TCR:CD3 sequestration observed in DP thymocytes suggested that there might be an active down-regulation process mediated by tonic CD3 ubiquitylation. Dynamin is a guanosine triphosphatase that is required for the fission of membrane and endocytic vesicles (Praefcke and McMahon, 2004). To determine whether dynamin contributes to TCR internalization in DP thymocytes, we treated neonatal thymi with a dynamin inhibitor (MiTMAB) (Figure 4A and B). There was a substantial increase of surface TCR on DP, but not SP thymocytes, suggesting that ubiquitylation may mediate enhanced, dynamin-dependent TCR:CD3 internalization and downregulation in immature DP but not mature SP thymocytes. However, additional studies will be required to fully elucidate the contribution of dynamin in these events.

The reduced CD3 protein levels in DP versus SP thymocytes, despite comparable mRNA levels (Supplementary Figure S1), suggested that the TCR:CD3 complex was being degraded in DP thymocytes. We reasoned that this Ub modification might mediate the internalization and trafficking of the TCR complex in DP thymocytes to sites of degradation; lysosomes or proteasomes. Thus, we next determined the fate of tonic ubiquitylated TCR:CD3 complexes on DP thymocytes in neonatal thymic organ culture experiments using inhibitors of lysosomal (NH_4Cl and Concanamycin A) and proteasomal degradation (MG132 and lactacystin). Consistent with earlier studies (Kosugi *et al*, 1992; Lacorazza and Nikolich-Zugich, 2004), our data suggest that the TCR:CD3 complexes in DP thymocytes are degraded in lysosomes, and not by proteasomes (Figure 4C). A recent study reported that a lysosomal protein, LAPTM5, controls the degradation of CD3ζ in DP thymocytes and activated T cells, a process that requires the LAPTM5 Ub-interacting motif (Ouchida *et al*, 2008). Consistent with these data, we observed that CD3ζ colocalized with LAPTM5 in DP but not SP thymocytes

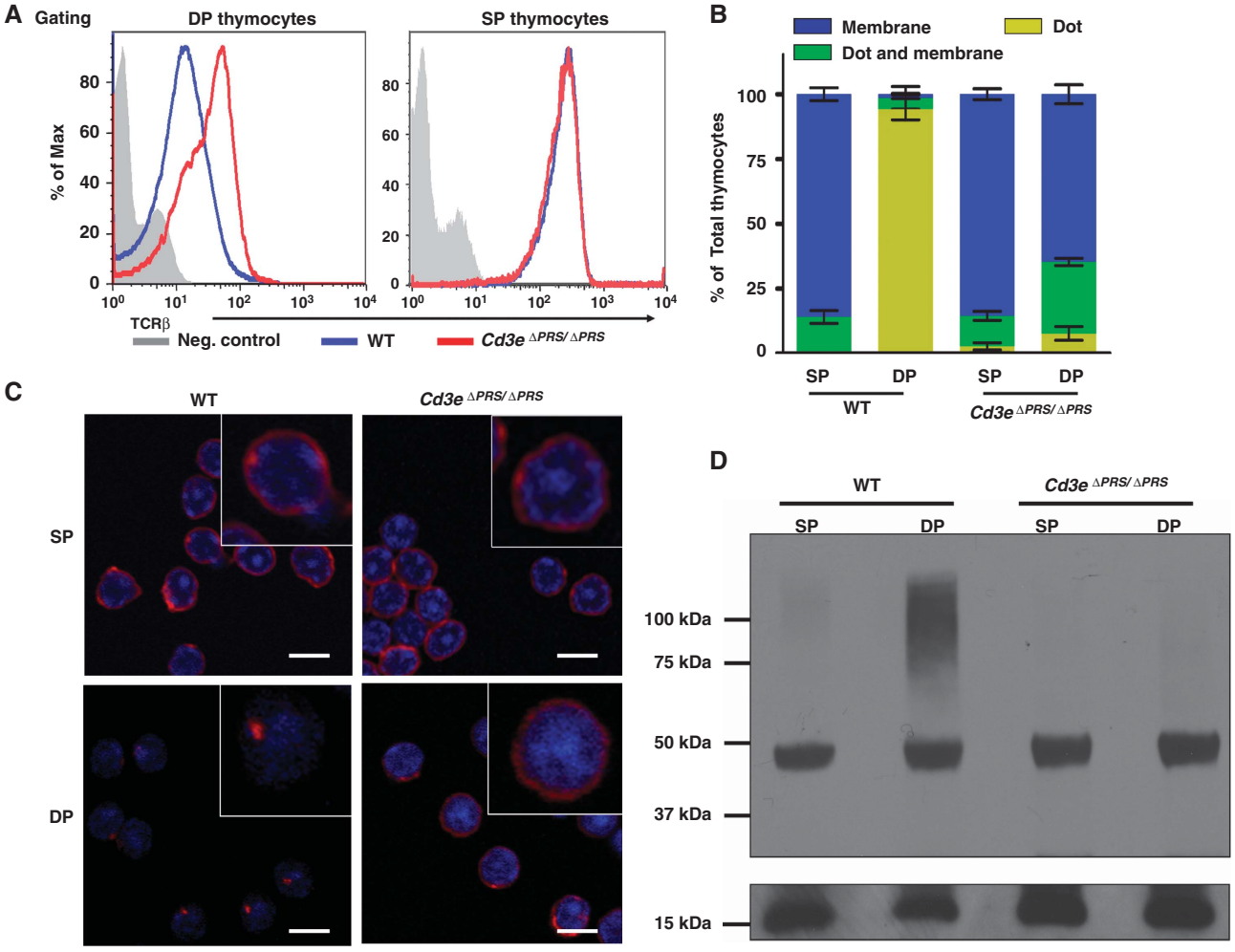

Figure 3 CD3ε-PRS mediates CD3 tonic ubiquitylation in DP thymocytes. (**A**) Thymi were isolated from WT or $CD3e^{\Delta PRS/\Delta PRS}$ mice. Surface expression of the TCR:CD3 complex was measured by flow cytometry on thymocytes co-stained with mAbs against CD4, CD8, and TCRβ. (**B**, **C**) Thymocytes were stained with anti-CD4 and anti-CD8, and DP and SP thymocytes were purified by FACS. Sorted thymocytes were fixed, permeabilized, and stained with Alexa-647-conjugated CD3ζ mAb. The distribution of CD3ζ in thymocyte populations is indicated ($n > 50$) (**B**). The localization of CD3ζ (red) and DAPI-stained nucleus (blue) are shown in representative confocal images (**C**). (**D**) Tonic ubiquitylation of CD3 was performed as described in Figure 1A.

(Supplementary Figure S3). Taken together, these data suggest that ubiquitylated TCR:CD3 complexes are degraded in lysosomes. However, additional analysis will be required to fully define the intracellular trafficking and degradation pathway of TCR:CD3 complexes in DP thymocytes.

Two types of Ub modification have been linked with protein internalization and trafficking to lysosomes; monoubiquitylation (Haglund *et al*, 2003a; Marmor and Yarden, 2004) and K63-linked polyubiquitylation (Mukhopadhyay and Riezman, 2007). To distinguish between these two forms of Ub modification, we used a mAb specific for all forms of poly-, but not mono-, ubiquitylated proteins (polyUb, clone FK1) (Haglund *et al*, 2003b; Fujimuro and Yokosawa, 2005) and a highly specific and sensitive K63-linked polyUb-specific mAb (K63Ub, clone HWA4C4) that we recently developed (Wang *et al*, 2008). Western blot analysis of CD3ζ in DP thymocytes clearly indicated that tonic Ub modification was detected with an mAb specific for Ub but not polyUb mAb or K63Ub mAb (Supplementary Figure S4). Taken together, these data are consistent with a model in which tonic monoubiquitylated TCR:CD3 complexes are internalized in a dynamin-dependent manner and transported to lysosomes for degradation, thus maintaining the characteristic low surface TCR expression seen on DP thymocytes. However, we cannot completely rule out the possibility that CD3 polyubiquitylation occurs, albeit to an extent that could not be detected with the approaches used here.

Tonic ubiquitylation regulates TCR surface expression on immature T cells

We next directly assessed whether this tonic ubiquitylation was required and sufficient for the regulation of TCR surface expression on DP thymocytes by generating mutant mice in which CD3 ubiquitylation was blocked. Of the known target residues for ubiquitylation (Cadwell and Coscoy, 2005), there are 37 lysines and no cysteines in the CD3 cytoplasmic domains (Figure 5A). Furthermore, it has been shown previously that the intracellular lysines of CD3ζ are required for CD3ζ ubiquitylation (Hou *et al*, 1994). To block CD3 tonic ubiquitylation, we generated mice expressing CD3 mutant molecules ($CD3^{KR}$), in which all cytoplasmic lysines were mutated to arginines, using retroviral-mediated stem cell

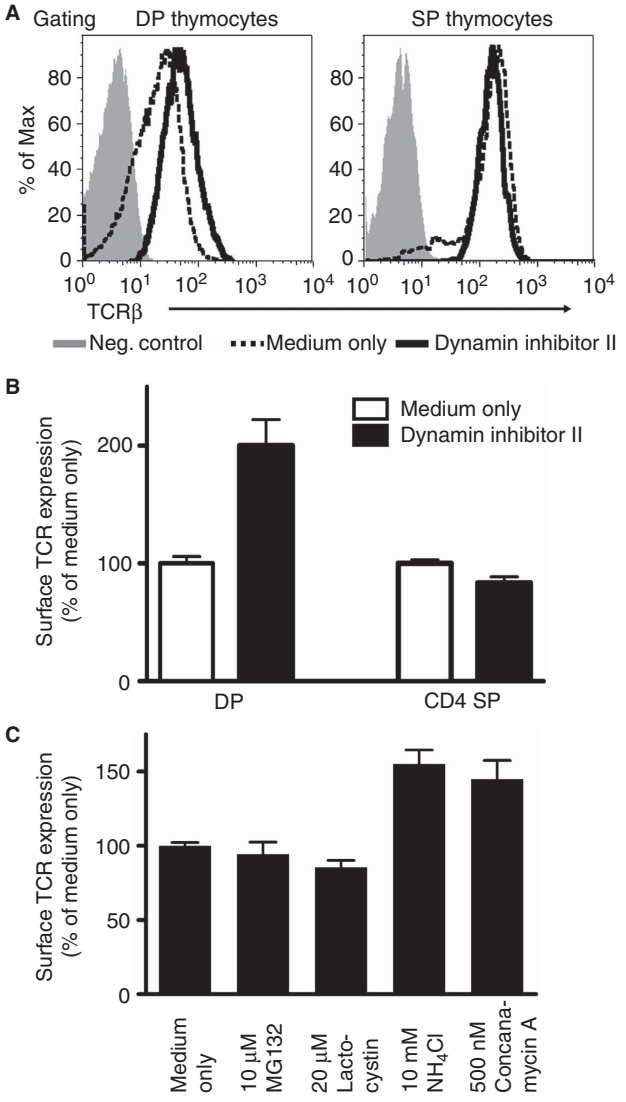

Figure 4 Monoubiquitylation induces dynamin-mediated internalization of the TCR:CD3 complex followed by lysosomal degradation. Thymic lobes from neonatal mice (P1) were treated with the inhibitors indicated for 20 h, and surface TCRβ expression determined by flow cytometry. Data represent the mean ± s.e.m. of 5–8 mice from 2 to 3 experiments per group (**A**, **B**) or 5–10 mice from 2 to 3 experiments per group (**C**). A full-colour version of this figure is available at *The EMBO Journal* Online.

gene transfer and multicistronic 2A peptide-linked retroviral vectors (Supplementary Figure S5) (Holst *et al*, 2006a, b) [referred to as retrogenic mice]. CD3-deficient bone marrow donors (referred to herein as CD3εζ$^{-/-}$ mice) were generated by crossing *Cd247*$^{-/-}$ (CD3ζ) and *Cd3e*$^{\Delta p/\Delta p}$ (CD3ε) mice. The latter possess a PGK-*neo* cassette inserted into *Cd3e* gene that results in the complete silencing of the adjacent *Cd3g* (CD3γ) and *Cd3d* (CD3δ) genes (Wang *et al*, 1999; Holst *et al*, 2008). *Rag1*$^{-/-}$ mice, which lack T and B cells, were used as recipients. Western blot analysis confirmed that CD3ζ was no longer ubiquitylated following activation of T cells from CD3^{KR} retrogenic mice (Figure 5B). Interestingly, TCR expression on CD3^{KR} DP thymocytes was upregulated more than three-fold compared with wild-type (CD3^{WT}) mice (Figure 5C). The upregulation of TCR on DP thymocytes from CD3^{KR} mice was not due to CD3 overexpression as the level of the green florescent protein (GFP) reporter contained within these retroviral vectors (an internal ribosome entry site-GFP) was comparable between the CD3^{WT} and CD3^{KR} retrogenic mice (Supplementary Figure S5C). In contrast to DP thymocytes, a 40% reduction in TCR expression was observed on SP thymocytes from CD3^{KR} mice compared with the CD3^{WT} mice, which is also observed in *Cbl*$^{-/-}$ and *Cbl* loss-of-function mutant mice (Thien *et al*, 2003). These data suggest that tonic CD3 ubiquitylation is required to control TCR expression on immature DP thymocytes.

Functional redundancy in the usage of ubiquitylated CD3 cytoplasmic lysines

All four CD3 cytoplasmic domains possess lysine residues (four in CD3γ, three in CD3δ, six in CD3ε, and nine in CD3ζ; Figure 5A). To determine which CD3 subunits contain functional ubiquitylation sites that are sufficient to limit TCR:CD3 expression on DP thymocytes, we generated four additional groups of retrogenic mice expressing combinations of one CD3 wild-type subunit with the remainder CD3^{KR} mutant (e.g. CD3ζ^{WT}δγε^{KR} encodes wild-type CD3ζ and lysine-mutated CD3δ, CD3γ, and CD3ε). TCR expression on DP thymocytes in the CD3δ^{WT}γεζ^{KR}, CD3ε^{WT}δγζ^{KR}, and CD3ζ^{WT}δγε^{KR} mice was restored to wild-type levels (Figure 5D). However, the CD3γ^{WT}δεζ^{KR} mice had higher TCR expression on DP thymocytes, indicating that the lysines in the CD3γ chain are insufficient for tonic ubiquitylation-meditated regulation of TCR expression on immature T cells. Therefore, there is functional redundancy in the usage of ubiquitylated CD3 cytoplasmic lysines and that very few lysines (3 lysines in CD3δ chain versus 37 in all the CD3 cytoplasmic domains) are sufficient to control TCR:CD3 expression.

CD3ζ monoubiquitylation is sufficient to mediate low TCR expression on DP thymocytes

Lysine residues can also be the target of other modifications, such as sumoylation, neddylation, and methylation, which may occur on CD3 molecules. None of these modifications were detectable on CD3ζ in either mature or immature T cells (Supplementary Figure S6), although we cannot exclude the possibility that increased TCR on DP thymocytes in CD3^{KR} mice is mediated by subtle or alternate CD3ζ modifications. Furthermore, we cannot completely rule out the possibility that these mutations may affect the structure of the CD3 cytoplasmic tails, although they appear relatively unstructured except for the ITAMs (Aivazian and Stern, 2000; Sigalov *et al*, 2006; Xu *et al*, 2008). They do appear to associate with the inner leaflet of the plasma membrane via electrostatic interactions between basic CD3 residues and acidic phospholipids, which one might predict would be retained with conservative Lys:Arg substitutions.

To address these issues directly, we investigated whether attaching Ub to the carboxy-terminal end of CD3ζ was able to restore control of TCR expression. We attached a monoUb to the COOH-terminus of CD3^{KR} (CD3^{KR}-monoUb) to mimic the monoubiquitylated form of the TCR:CD3 complex. Two modifications were made to ensure that this Ub moiety was not further modified. First, all seven Ub lysines were mutated to arginine (K6/11/27/29/33/48/63R) to prevent polyubiquitylation (Shih *et al*, 2000). Second, the two C-terminal glycine residues (G75/76) were removed to prevent conjugation to endogenous Ub or another protein. We assessed TCR:CD3

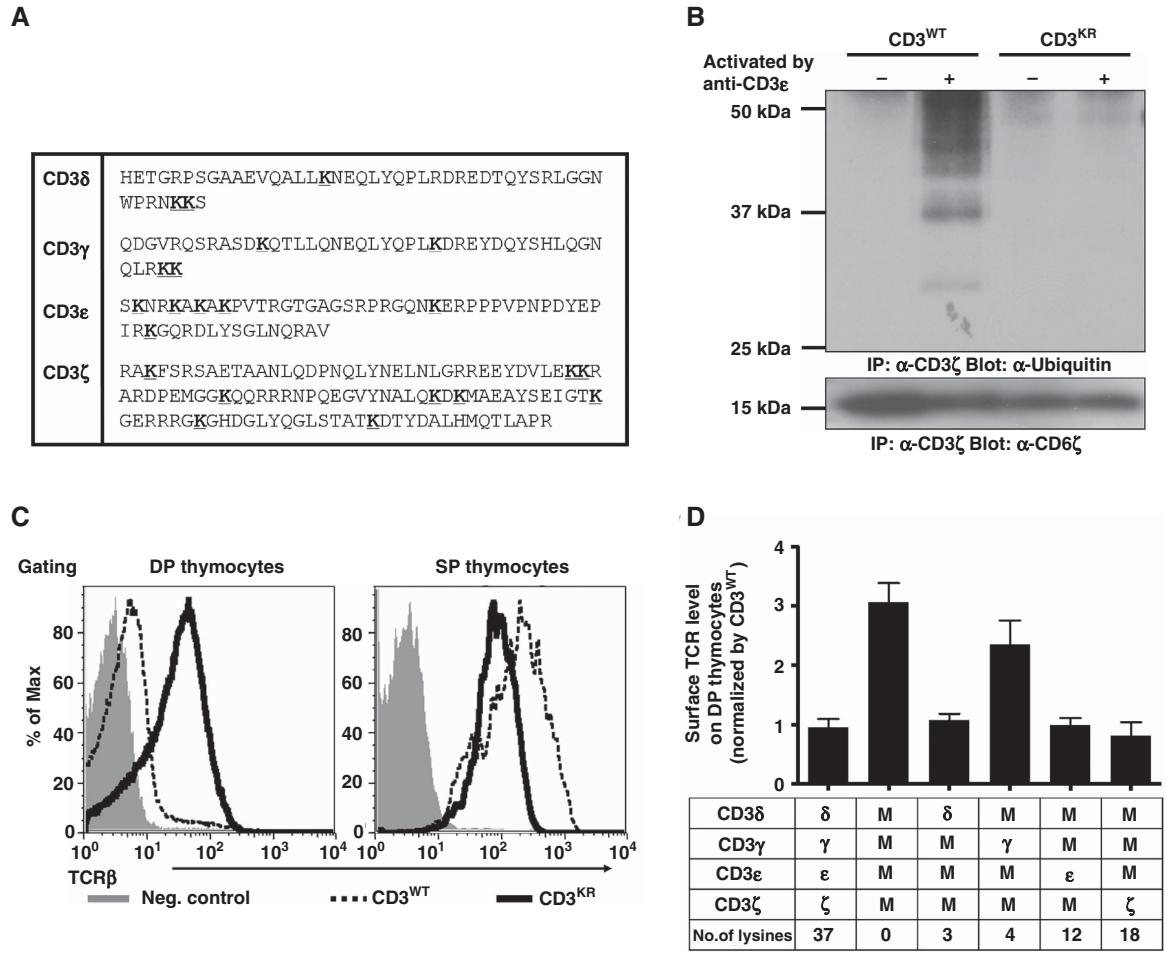

Figure 5 Regulation of TCR surface expression by CD3 tonic ubiquitylation. (**A**) The amino-acid sequence of the CD3 cytoplasmic domains highlighting the 37 lysine residues (bold and underlined). (**B**) Retrogenic mice were generated by transducing CD3εζ$^{-/-}$ bone marrow with CD3^{WT} or CD3^{KR} constructs as indicated, transplanting into sublethally irradiated *Rag1*$^{-/-}$ recipients and analysing 5–8 weeks after transfer. Splenic T cells were purified by MACS from either CD3^{WT} or CD3^{KR} retrogenic mice and activated by CD3ε mAb. Lysates were immunoprecipitated with CD3ζ mAb, separated by SDS–PAGE, and western blots were probed with ubiquitin mAb as in Figure 1A. The membrane was stripped and re-probed with CD3ζ mAb to show comparable CD3ζ loading. (**C**) Representative flow cytometry histograms are presented showing surface TCRβ expression on DP and SP thymocytes from CD3^{WT} and CD3^{KR} retrogenic mice (representative of 10–20 mice from more than three experiments). (**D**) Surface TCRβ expression on DP thymocytes of CD3^{WT} or various CD3^{KR} retrogenic mice, determined by flow cytometry. The difference between CD3^{WT} and CD3δεζ^{KR}γ^{WT} is statistically significant ($P = 0.008$). Data represent the mean ± s.e.m. of 5–20 mice from more than two experiments. A full-colour version of this figure is available at *The EMBO Journal* Online.

complex expression on HEK-293T cells using CD3ζ^{KR}, CD3ζ^{KR}-monoUb, or an unmodified Ub attached to the CD3ζ^{KR} COOH-terminus as a control (CD3ζ^{KR}-Ub). Attaching either form of Ub to CD3ζ substantially reduced TCR expression (Supplementary Figure S7A).

As the internalization of ubiquitylated TCR:CD3 appeared to be dynamin-dependent (Figure 4A and B), and enzymatically inactive forms of dynamin act as dominant negatives and block endocytosis (Praefcke and McMahon, 2004), we reasoned that such mutants should block the internalization of an appropriately modified CD3-Ub chimera. Dominant negative dynamin had no effect on TCR expression on the CD3ζ^{KR}-Ub transfectants, suggesting that attachment of wild-type Ub to CD3ζ^{KR} led to intracellular TCR:CD3 complex retention and/or degradation (Supplementary Figure S7B). In contrast, normal expression of TCR complexes containing CD3ζ^{KR}-monoUb was completely restored by dominant negative dynamin, consistent with our observations using the dynamin inhibitor MiTMAB with DP thymocytes. Collectively, these data suggested that CD3 monoubiquitylation triggered rapid dynamin-dependent TCR internalization and lysosomal sequestration.

Retrogenic mice expressing CD3^{KR}-monoUb showed a 50% reduction in TCR:CD3 complex expression on DP thymocytes compared with CD3^{WT} levels, showing that 'increased' ubiquitylation of CD3ζ on DP thymocytes leads to reduced surface TCR expression (Figure 7A). Collectively, these data are consistent with a model in which highly controlled, tonic ubiquitylation is required for fine tuning TCR:CD3 complex expression on immature DP thymocytes.

Tonic ubiquitylation controls TCR signalling in DP thymocytes and regulates T-cell development

To test whether altered tonic ubiquitylation of the TCR:CD3 complex affects TCR signalling in DP thymocytes, we measured immunological synapse (IS) formation and ERK phosphorylation status in activated CD3^{WT} and CD3^{KR} DP thymocytes. Earlier studies have suggested that DP thymocytes

form multifocal synapses, whereas mature peripheral T cells form compact IS (Hailman *et al*, 2002). Thus, we used total internal reflection fluorescent microscopy with synthetic planar lipid bilayer containing unlabelled His-tagged ICAM-1 and fluorescently labelled streptavidin conjugated to biotinylated TCRβ mAbs to assess the effect of abrogated CD3 ubiquitylation on IS formation in DP thymocytes. Significant differences were observed between the pattern of IS formed by $CD3^{WT}$ versus $CD3^{KR}$ DP thymocytes 15 min poststimulation on lipid bilayers. First, the TCR contact area was significantly reduced with $CD3^{KR}$ DP thymocytes (Figure 6A). Second, the percentage of compact IS relative to multifocal and diffuse IS was significantly increased with $CD3^{KR}$ versus $CD3^{WT}$ DP thymocytes (Figure 6B and C). These data suggest that preventing CD3 ubiquitylation leads to IS formation that is more reminiscent of mature peripheral T cells.

We also assessed ERK phosphorylation following TCR stimulation of DP thymocytes by flow cytometry. A significantly increased percentage of $CD3^{KR}$ versus $CD3^{WT}$ DP thymocytes expressed pERK (Figure 6D and E). Similar observations were also made with $Sla^{-/-}$ DP thymocytes (Supplementary Figure S8). The specificity of these observations was confirmed by the use of the MEK inhibitor U0126. Interestingly, increased pERK was more prolonged in $CD3^{KR}$ DP thymocytes compared with $Sla^{-/-}$ thymocytes (Figure 6D; Supplementary Figure S8A). Taken together, these data suggest that blocking CD3 tonic ubiquitylation increases TCR signalling strength in immature T cells.

To determine whether tonic ubiquitylation of the TCR:CD3 complex regulates T-cell development, we analysed the distribution of DN, DP, and SP thymocytes and total thymocyte number in $CD3^{WT}$, $CD3^{KR}$, and $CD3^{KR}$-monoUb retrogenic mice. $CD3^{KR}$ mice had twice as many thymocytes as $CD3^{WT}$ mice, whereas $CD3^{KR}$-monoUb mice had substantially reduced thymic cellularity that was ~1/10th of $CD3^{WT}$ mice (Figure 7B). The distribution of DN, DP, and SP thymocytes in $CD3^{KR}$ versus $CD3^{WT}$ mice was unchanged (Figure 7C and D; Supplementary Figure S9A). In contrast, $CD3^{KR}$-monoUb

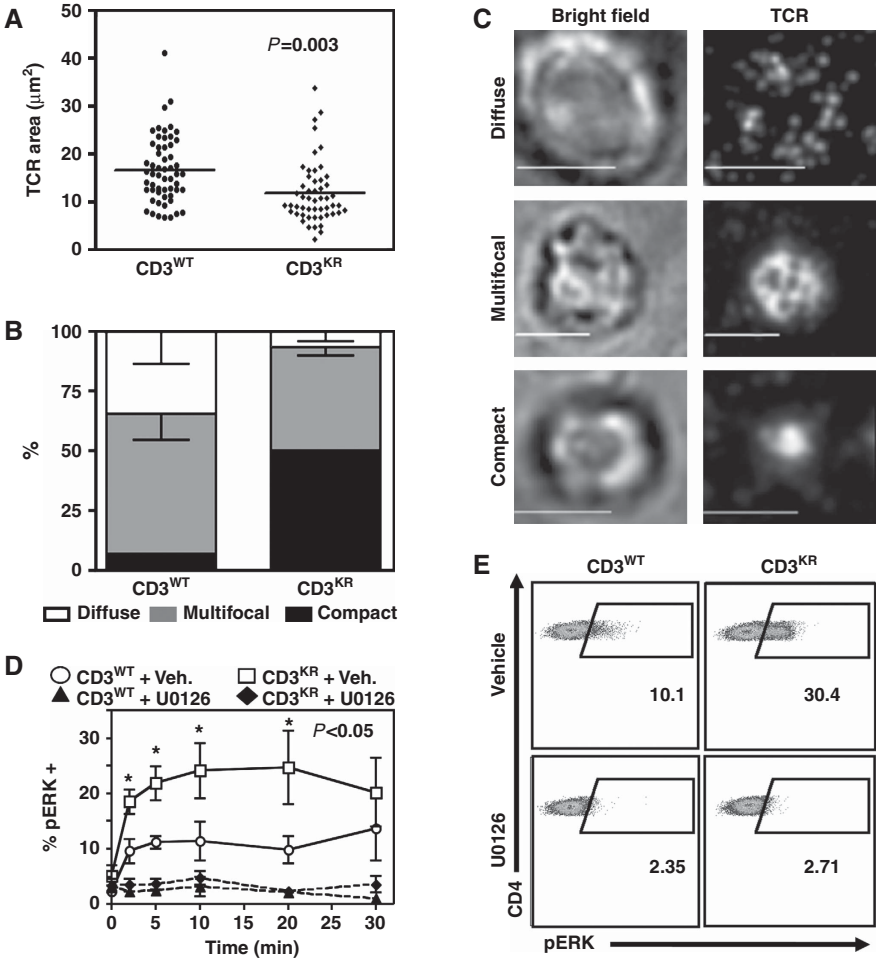

Figure 6 Impaired CD3 ubiquitylation enhances IS formation and increases ERK phosphorylation in DP thymocytes. (**A**–**C**) DP thymocytes from $CD3^{WT}$ or $CD3^{KR}$ Rg mice were purified by FACS and added to a synthetic planar lipid bilayer containing unlabelled His-tagged ICAM-1 and fluorescently labelled streptavidin conjugated to biotinylated anti-TCRβ antibodies. Interactions were fixed following 15 min stimulation, visualized using spinning disk confocal microscopy and analysed using Slidebook software. (**A**) The area of individual DP T-cell synapses obtained in two independent experiments. (**B**, **C**) The morphology of individual synapses were classified according to the representative images (**B**) and shown graphically (**C**—Scale bar = 5 mm). (**D**, **E**) Thymocytes from $CD3^{WT}$ or $CD3^{KR}$ Rg mice were isolated, rested in 0.5% FCS RPMI for 1 h at 37°C, and then activated by crosslinking using a CD3ε mAb in the presence (+U0126) or absence (+Veh.) of the MEK inhibitor U0126. Thymocytes were then fixed at the indicated time, permeablized, and stained with CD4, CD8, and pERK Abs. (**D**) CD4 + CD8 + DP thymocytes were gated and pERK expression measured. (**E**) Representative flow cytometry dot plots are shown 10 min after stimulation. Statistical significance was determined using an unpaired *t* test in Prism software.

mice had an increased percentage of DN thymocytes (Figure 7C and D). Furthermore, the number and percentage of natural regulatory T cells (T_{regs}) in the thymus and spleen was substantially reduced in $CD3^{KR}$-monoUb mice (Figure 7E–G). Interestingly, the $CD3^{KR}$-monoUb mice became sick and died 8–12 weeks post bone marrow transfer, with moderate inflammation, multifocal crypt necrosis, and enterocyte apoptosis in the small and large intestines, and vasculitis in the liver (Supplementary Figure S10 and Supplementary Table S1). Whether this disease is due to a breakdown of central tolerance as described in other mouse models with TCR hyporesponsiveness (Aguado *et al*, 2002; Sommers *et al*, 2002; Holst *et al*, 2008) and/or limited generation of natural T_{regs} in the periphery is unclear and would require further analysis. To rule out the possibility that hypo- or hyper-ubiquitylation promotes the generation of DP thymocytes in the absence of TCR, we generated retrogenic mice using $CD3^{WT}$, $CD3^{KR}$, and $CD3^{KR}$-monoUb transduced $CD3\varepsilon\zeta^{-/-}$ $Rag1^{-/-}$ bone marrow (Supplementary Figure S11). In the absence of TCR, we found little, if any, DP thymocytes in $CD3^{WT}$, $CD3^{KR}$, or $CD3^{KR}$-monoUb retrogenic mice. Collectively, these results suggest that the altered TCR:CD3 ubiquitylation in thymocytes affects T-cell development.

Finally, to assess whether positive and negative selection were altered by the extent of TCR:CD3 ubiquitylation, we generated retrogenic mice co-expressing either $CD3^{WT}$, $CD3^{KR}$, or $CD3^{KR}$-monoUb with the MataHari TCR, a H-$2K^{b}$-restricted $V\beta8.3^{+}$ TCR specific for the male antigen Uty (Valujskikh *et al*, 2002; Holst *et al*, 2006b). Bone marrow from male or female $CD3\varepsilon\zeta^{-/-}Rag1^{-/-}$ mice was transduced with retrovirus encoding the MataHari TCR and the different versions of CD3, and transplanted into irradiated $Rag1^{-/-}$ mice of the same sex. In female mice, where MataHari T cells are normally positive selected, we observed a uniform population of $CD8^{+}V\beta8.3^{+}$ T cells in the periphery in mice expressing $CD3^{WT}$ molecules (Figure 8A). However, $CD3^{KR}$ female mice have ~3 times more thymocytes than $CD3^{WT}$ female mice, a higher CD8 SP to TCR^{+} DP thymocyte ratio, and a higher percentage of $CD8^{+}V\beta8.3^{+}$ T cells in the periphery (Figure 8A and B; Supplementary Figure S9B), suggesting that positive selection was enhanced when CD3 tonic ubiquitylation was blocked. Interestingly, T-cell development was blocked at the DP stage in MataHari $CD3^{KR}$-monoUb female mice with very low CD5 expression on DP thymocytes, normally upregulated on positive selection (Jameson *et al*, 1995; Tarakhovsky *et al*, 1995), and no T cells found in the periphery, despite substantial thymocyte

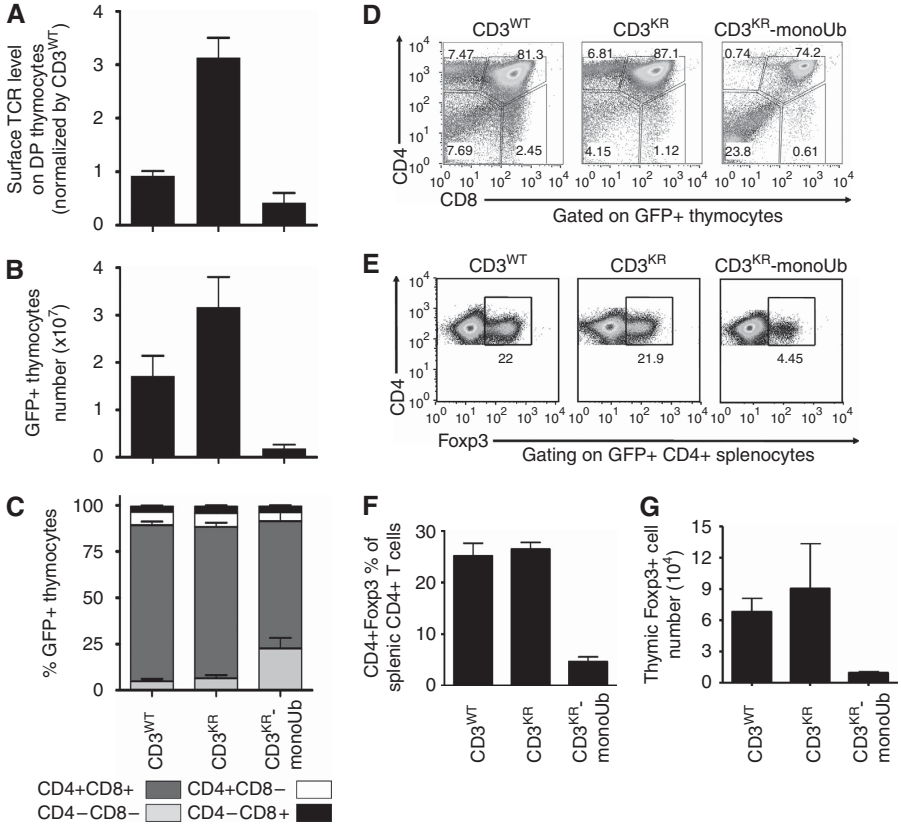

Figure 7 CD3 ubiquitylation alters TCR expression, thymic cellularity, and T_{reg} development. Retrogenic mice were generated by reconstituting sublethally irradiated $Rag1^{-/-}$ recipients with transduced $CD3\varepsilon\zeta^{-/-}$ bone marrow. Mice were analysed 5–8 weeks after transfer. Thymocytes were counted and stained with antibodies to CD4, CD8, and TCRβ, and analysed by flow cytometry. Surface TCR level on GFP^{+} DP thymocytes (**A**), GFP^{+} thymocyte number (**B**), and the percentage of GFP^{+} thymocytes (**C**) was determined. Data were gated on live, GFP^{+} cells, and represent the mean ± s.e.m. of 10–20 mice from more than three experiments per group. (**D**) Representative dot plots of GFP^{+} thymocytes stained with antibodies to CD4 and CD8 are shown (representative of 10–20 mice from more than three experiments). (**E–G**) Splenocytes and thymocytes were surface stained with CD4 mAb and intracellularly stained with Foxp3 mAb. Representative dot plots of $CD4^{+}$ splenic T cells from a retrogenic mouse experiment are shown (**E**). Bar charts show the percentage of splenic $CD4^{+}$ T-cells expressing Foxp3 (**F**) and the number of $Foxp3^{+}$ thymocytes (**G**). A full-colour version of this figure is available at *The EMBO Journal* Online.

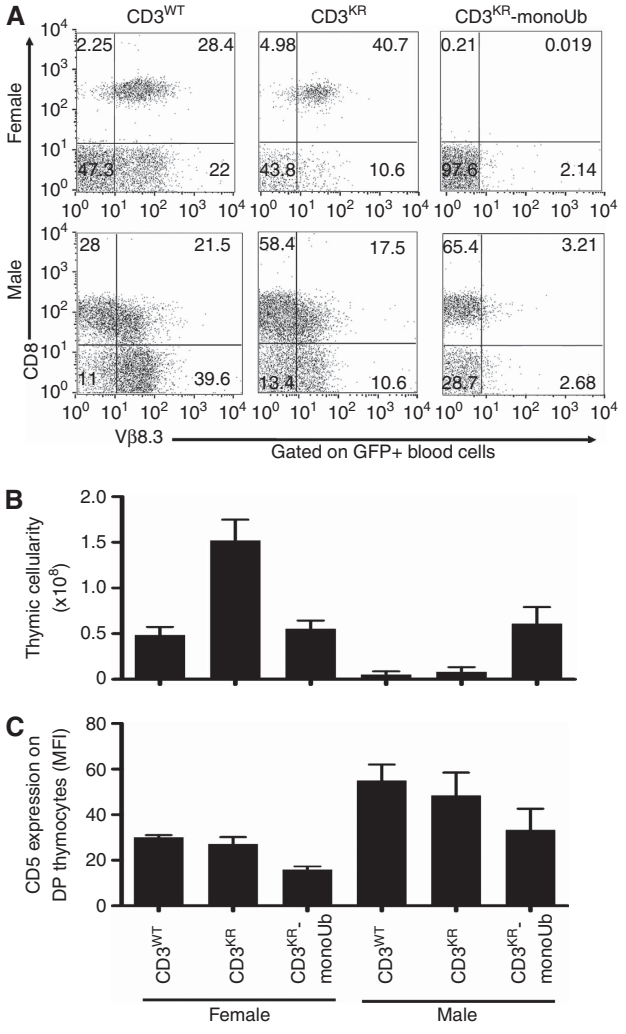

Figure 8 TCR:CD3 ubiquitylation modulates T-cell development. (**A–C**) Bone marrow from female or male CD3ζε$^{-/-}$*Rag1*$^{-/-}$ mice was co-transduced with the MataHari TCRαβ and CD3^{WT}, CD3^{KR}, or CD3^{KR}-monoUb. Transduced bone marrow was used to reconstitute irradiated, sex-matched *Rag1*$^{-/-}$ mice. Mice were bled and killed 6–8 weeks after transfer. Peripheral blood was stained with antibodies to CD8 and Vβ8.3 (**A**). Bar charts show the number of thymocytes (**B**) and CD5 expression on DP thymocytes (**C**) in each group. Data are gated on GFP$^+$ cells and are represent the mean ± s.e.m. of 5–10 mice from two experiments per group. A full-colour version of this figure is available at *The EMBO Journal* Online.

cellularity (Figure 8A–C; Supplementary Figure S12). These data suggest that the signal that normally mediates positive selection in MataHari mice may be converted into one that mediates neglect in CD3^{KR}-monoUb MataHari female mice (Supplementary Figure S13).

In male mice, CD3^{WT} and CD3^{KR} MataHari T cells were deleted normally, as expected, with thymic cellularity significantly reduced compared with MataHari female mice (Figure 8A–C; Supplementary Figure S12). As shown earlier, some T cells escaped negative selection in the thymus by downregulation of CD8 and/or TCR expression and appear in the periphery (Figure 8A) (Valujskikh *et al*, 2002). It is noteworthy that in both male and female CD3^{KR} MataHari mice, there are significantly less CD8$^-$Vβ8.3$^+$ T cells in the periphery (Figure 8A), again suggesting that altered CD3 ubiquitylation can alter T-cell tolerance mechanisms. In contrast, there appeared to be no deletion in male CD3^{KR}-monoUb MataHari mice as thymus size and cellularity, and CD5 expression on DP thymocytes were comparable to CD3^{WT} female mice (Figure 8B and C; Supplementary Figure S12), and a uniform population of GFP$^+$CD8$^+$ T cells was found in the blood (Figure 8A) [note that consistent with CD3^{KR}-monoUb mice, TCR surface expression is also low in the MataHari CD3^{KR}-monoUb mice]. These data suggest that negative selection may be converted into positive selection in CD3^{KR}-monoUb MataHari male mice (Supplementary Figure S13). Collectively, these findings suggest that the signalling threshold for positive and negative selection is directly regulated by tonic ubiquitylation of the TCR:CD3 complex.

Discussion

Our results show that c-Cbl-dependent, MHC-independent constitutive, tonic ubiquitylation of the TCR:CD3 complex occurs in, and is restricted to, DP thymocytes. Earlier studies had suggested that TCR:CD3 ubiquitylation was restricted to activated mature T cells (Cenciarelli *et al*, 1992). CD3 tonic ubiquitylation is mediated by Lck and the CD3ε-PRS, which may cooperatively recruit a c-Cbl:SLAP complex that subsequently mediates CD3 ubiquitylation. We further show that CD3 tonic ubiquitylation is required and sufficient for the downregulation of surface TCR expression, a hallmark of T-cell development. Our data are consistent with a model in which there is a moderate level of constitutive Lck activity, perhaps facilitated by the miR181a-mediated depletion of Src-inactivating phosphatases (Li *et al*, 2007b), which phosphorylates CD3 and the c-Cbl:SLAP complex that is subsequently recruited to the CD3ε-PRS, perhaps via Nck. Collectively, these molecular events mediate the tonic ubiquitylation of the TCR:CD3 complex in the absence of any TCR:MHC ligation. The ubiquitylated TCR:CD3 complex is internalized in a dynamin-dependent manner and transported to lysosomes for degradation mediated by LAPTM5. Clearly, there are steps in this model that require further examination. First, whether there is a direct connection between miR181a and tonic ubiquitylation remains to be determined. Second, whether Lck is directly or indirectly recruited to the CD3ε PRS by Nck remains to be determined. Indeed, the precise role of the CD3ε PRS in mediating tonic ubiquitylation remains obscure. Third, the precise fate and pathway followed by tonic ubiquitylated TCR:CD3 complexes remains to be more fully defined.

Why might tonic ubiquitylation only occur in DP thymocytes? First, the E3 ligase machinery that drives tonic ubiquitylation (c-Cbl + Slap) is predominantly expressed in immature T cells (Sosinowski *et al*, 2001; Molero *et al*, 2004; Myers *et al*, 2006). Although c-Cbl is expressed at higher amounts in thymocytes, compared with splenic T cells, SLAP expression is essentially restricted to DP thymocytes, which may serve to mitigate c-Cbl activity. Second, a recent study has shown that high expression of miR181a in DP thymocytes represses multiple phosphatases, which leads to increased tyrosine kinase activity and target phosphorylation (Li *et al*, 2007b). This may in turn lead to enhanced activation of the c-Cbl:SLAP complex.

The requirement for a c-Cbl:SLAP complex as the primary, if not sole mediator of TCR:CD3 tonic ubiquitylation, is

consistent with earlier studies showing that *Cbl*$^{-/-}$ and *Sla*$^{-/-}$ mice have upregulated TCR expression on DP thymocytes and increased positive selection in DO11.10 TCR transgenic mice (Naramura *et al*, 1998; Sosinowski *et al*, 2001; Myers *et al*, 2006). Importantly, we also show that the modulation of TCR:CD3 ubiquitylation has a substantial effect on thymocyte cellularity, positive and negative selection, and ultimately the establishment of a peripheral T-cell pool. We propose that tonic ubiquitylation of the TCR:CD3 complex on immature thymocytes tightly regulates surface TCR expression and may help to maintain the fidelity of T-cell development.

How might the CD3ε-PRS be involved in mediating CD3 tonic ubiquitylation in DP thymocytes? Our data have shown that Lck is required for tonic ubiquitylation of CD3 in immature T cells. A recent study proposed that recruitment of Lck in immature T cells is mediated by the CD3ε-PRS in DP thymocytes (Mingueneau *et al*, 2008). The CD3ε-PRS seems to be constitutively exposed and interacts with Nck (Gil *et al*, 2002; Mingueneau *et al*, 2008; Brodeur *et al*, 2009; Wang *et al*, 2009). As ~50% of Lck in DP thymocytes is not associated with CD4 or CD8, this free Lck may be recruited to the TCR:CD3 complex by interacting with the Nck-SH3 domain, resulting in tonic phosphorylation of CD3 chains, c-Cbl and SLAP, which in turn triggers the tonic CD3 ubiquitylation pathway. Consistent with this model, our data showed that tonic CD3 ubiquitylation is blocked and the distribution of the TCR:CD3 is altered in DP thymocytes from mice lacking CD3ε-PRS, Lck, c-Cbl, or SLAP.

In addition to observing increased TCR on DP thymocytes in CD3^{KR} mice, counterintuitively, we also saw a reduction in TCR expression on SP thymocytes and peripheral T cells (Figure 5C). Interestingly, similar observations were made with the *Cbl*$^{-/-}$ or *Cbl* loss-of-function mutant mice (Thien *et al*, 2003). This phenotype may be cell extrinsic and caused by increased TCR signalling strength due to higher TCR expression on DP thymocytes (Figure 6; Supplementary Figure S8), which may lead to increased negative selection. Thus, some thymocytes, which have lower TCR expression, may successfully escape negative selection. Alternatively, lowered TCR on SP and peripheral T cells, may be cell intrinsic with ubiquitylation performing a protective role in the regulation of TCR expression in mature T cells, perhaps by limiting AP1-mediated internalization (Liu *et al*, 2000; Szymczak and Vignali, 2005).

Several lines of evidence suggest that the primary role of CD3 ubiquitylation in DP thymocytes is to target the TCR:CD3 complex to lysosomes for degradation thereby reducing surface TCR expression. First, earlier studies have shown that receptor monoubiquitylation mediates their internalization and lysosomal degradation (Shih *et al*, 2000; Haglund *et al*, 2003b). Our data suggest that the TCR:CD3 complex in DP thymocytes is constitutively subjected to multiple monoUb additions to multiple lysine residues throughout the CD3 cytoplasmic domains. Second, we have also shown that mimicking monoubiquitylation by expression of a CD3ζ-monoUb chimeric molecule reduces surface TCR expression on thymocytes. Third, blocking internalization with dynamin inhibitors (MiTMAB) or blocking lysosomal degradation with acidification inhibitors (NH_4Cl or Concanamycin A) resulted in increased surface TCR expression on DP thymocytes. Fourth, a recent study showed that LAPTM5 mediates CD3 degradation in both thymocytes and activated T cells (Ouchida *et al*, 2008). Interestingly, the Ub-interacting motif of LAPTM5 is required for CD3 degradation. Our data showed that LAPTM5 and CD3 colocalize in immature DP thymocytes, which is consistent with a model in which LAPTM5 may selectively interact with ubiquitylated CD3 via its Ub interacting motif to induce CD3 lysosomal degradation. At odds with this model is the observation that TCR expression on DP thymocytes was only slightly increased in *Laptm5*$^{-/-}$ mice, in contrast to the significant increases seen with *Cbl*$^{-/-}$, *Sla*$^{-/-}$, *Lck*$^{-/-}$, and *Cd3e*$^{\Delta PRS/\Delta PRS}$ mice (Figures 1G, 2C, and 3B; Supplementary Figure S2B) (Molina *et al*, 1992; Sosinowski *et al*, 2001; Molero *et al*, 2004; Mingueneau *et al*, 2008; Ouchida *et al*, 2008). However, it is possible that other Ub-binding, lysosomal resident proteins cooperate with LAPTM5 to mediate TCR:CD3 sequestration and degradation.

Why might tonic CD3 ubiquitylation in DP thymocytes be important? Clearly, a key consequence of abrogating tonic CD3 ubiquitylation is increased TCR. Our data suggest that this leads to altered IS formation and contraction, and increased Erk phosphorylation, thereby shifting the signalling threshold for positive and negative selection, and regulatory T-cell development. Earlier studies have suggested that the type of synapse that forms between DP thymocytes and thymic epithelial cells (TECs) alters their selection fate (Hailman *et al*, 2002; Richie *et al*, 2002; Ebert *et al*, 2008). Thus, tonic ubiquitylation may help to ensure that the appropriate DP:TEC interactions are maintained for optimal positive and negative selection. What remains unclear is whether modulated IS formation and signalling are a direct consequence of increased TCR or are independently regulated by tonic CD3 ubiquitylation.

Understanding the role of CD3 tonic ubiquitylation in immature T cells may also provide insight into the role/contribution of CD3 ubiquitylation in activated T cells, whether this is mediated by the same, related or distinct molecules, and if the fate of ubiquitylated CD3 following TCR ligation is the same or different. Our results suggest that disruption of TCR expression control in DP thymocytes can lead to increased TCR signalling, altered positive and negative selection, and reduced T_{reg} development. Although profound autoimmunity was not observed in CD3^{KR} mice, it is possible that more chronic conditions may develop with time as has been observed in mice possessing mutations that disrupt TCR expression (Naramura *et al*, 2002). Clearly, this is a topic that will require further study.

Materials and methods

Mice

Rag1$^{-/-}$ (recombination activating gene 1) and C57BL/6J mice were obtained from Jackson Laboratories. *B2m*$^{-/-}$*Abb*$^{-/-}$ mice (β-2 microglobulin and MHC class II H-2A β chain, respectively) were purchased from Taconic Farms. Thymi from *Cbl*$^{-/-}$ (c-Cbl—Casitas B-lineage lymphoma) mice were provided by M Naramura and H Band. Thymi from *CD3e*$^{\Delta PRS/\Delta PRS}$ mice were provided by B Malissen. Thymi from *Lck*$^{-/-}$ and *Fyn*$^{-/-}$ mice were provided by S Hayes. Thymi from *Slap*$^{-/-}$ mice were provided by L Dragone. CD3εζ-KO mice lacking all four CD3 chains were generated by crossing *CD3e*$^{\Delta P/\Delta P}$ mice with *CD3ζ*$^{-/-}$ mice as described earlier (Wang *et al*, 1999; Szymczak *et al*, 2004; Holst *et al*, 2008). All animal experiments were performed in an AAALAC-accredited, Helicobacter-free, specific pathogen-free facility following national, state, and institutional guidelines. Animal protocols were approved by St Jude Institutional Animal Care and Use Committee.

Generation of CD3 multicistronic vectors
See Supplementary data.

Generation of retroviral producer cells
See Supplementary data.

Retroviral-mediated stem cell gene transfer
Retroviral transduction of murine bone marrow cells was performed as described earlier (Holst *et al*, 2006a, b, 2008). Briefly, we harvested bone marrow from 8- to 10-week-old donor mice 48 h after treatment with 150 mg/kg 5-fluoruracil (Pharmacia & Up-John). Bone marrow cells were single cell suspended and cultured in complete DMEM with 20% fetal bovine serum (FBS), murine IL-3 (20 ng/ml), human IL-6 (50 ng/ml), and murine SCF (50 ng/ml) (Biosource-Invitrogen) for 48 h. Cells were subsequently co-cultured for a further 48 h with irradiated (1200 rads) retroviral producer cell lines plus polybrene (6 μg/ml) and cytokines as detailed above. Transduced bone marrow cells were collected, washed, and cells resuspended in phosphate-buffered saline (PBS) containing 2% FBS with heparin (20 U/ml). Bone marrow cells (4×10^6 per mouse) were injected via the tail vein into irradiated (450 rads) *Rag1*$^{-/-}$ recipient mice. Retrogenic mice were analysed 6–12 weeks after bone marrow transplant.

Flow cytometric analysis, intracellular staining, and cell sorting
See Supplementary data.

CD3ε crosslinking and intracellular staining
See Supplementary data.

Confocal microscopy
Thymocytes purified by fluorescence-activated cell sorting (FACS) were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. These fixed cells were plated to the chamber of the glass slide (Nunc), which was pretreated with 0.1% Polyethyleneimine solution (Sigma). After 1 h incubation at room temperature, the chamber slide was washed twice with PBS and then treated with Image-iT FX signal enhancer (Invitrogen) for 30 min. Primary Abs in 2% milk solution were added into the chamber and the slide was kept at 4°C over night. The slide was washed extensively with PBS, and fluorchrome labelled secondary Abs diluted in 2% milk solution were added into the chamber and kept at room temperature for 1 h. After washing the chamber four times with PBS, the stained cells were mounted using Prolong Gold antifade reagent with DAPI (Invitrogen). All images were taken using a Zeiss LSM 510 NLO META confocal microscope. CD3ζ (H146) mAb was conjugated with Alexa-647 using an Alexa Fluor labelling kit (Invitrogen) following the manufacturer's instructions. LAPTM5 mAb was provided from Ji-yang Wang, RIKEN, Yokohama, Japan. An Alexa-488-conjugated mAb against rabbit IgG was purchased from Invitrogen.

Lipid bilayers and microscopic analyses
See Supplementary data.

RNA, cDNA, and quantitative real-time PCR
See Supplementary data.

Immunoprecipitation and western blotting
Immunoprecipitation and immunoblotting were performed as described earlier (Li *et al*, 2007a). Briefly, cells were lysed with lysis buffer containing 1% Triton X-100, 50 mM Tris–HCl (pH 7.6), 300 mM NaCl, 2 mM Pefabloc, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 1 μg/ml Pepstatin (Roche), and 10 mM *N*-ethylmaleimide (Sigma) on ice for 30 min, followed by centrifugation at 15 000 *g* for 15 min. For protein analysis, 20 μl of lysate was collected, gel separated, and immunoblotted with indicated antibodies. Antibodies against the following proteins were used: CD3ε (HMT3.1 mAb) (Szymczak *et al*, 2004), CD3ζ (H146-968 mAb, gift from Ralph Kubo, Cytel Corp., San Diego, CA) (Liu *et al*, 2000), α-Tubulin (Sigma), CD3δ and CD3γ (two rabbit polyclonal antisera that were generated in our laboratory) (Holst *et al*, 2008). For immunoprecipitation, 1 ml lysate was precleared with protein G-sepharose beads and further immunoprecipitated with a polyclonal antisera against CD3ζ (551-ζ, gift from David Wiest, Fox Chase Cancer Center, Philadelphia, PA) conjugated Protein G-sepharose beads. Immunoprecipitates were resolved by SDS–PAGE (Invitrogen Life Technologies), and blots were probed with mAbs against Ub (P4D1; Santa Cruz), polyUb (FK1, Millipore), K63Ub (HWA4C4; Wang H, submitted for publication), Sumo-1 (ZYMED), Nedd8 (gift from Brenda Schulman, SJCRH, Memphis, TN), or pan methyl lysine (Abcam). Blots were developed using enhanced chemiluminescence (Amersham Biosciences) and autoradiography. Membranes were stripped and blots re-probed with CD3ζ mAb (H146-968).

Transient transfection of HEK-293T cells
See Supplementary data.

Thymic organ culture
See Supplementary data.

Histological examination
See Supplementary data.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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